UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION OF PROTEIN KINASE INHIBITORY ACTIVITIES FROMSTREPTOMYCES STRAIN H7372 FOR POTENTIAL USE AS ANTI-CANCERAGENT

MASRIANA HASSAN

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IDENTIFICATION OF PROTEIN KINASE INHIBITORY ACTIVITIES FROM *STREPTOMYCES* STRAIN H7372 FOR POTENTIAL USE AS ANTI-CANCER AGENT

By

MASRIANA HASSAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfillment of the Requirement for the Degree of Master of Science

February 2007

Specially dedicated to,

My husband, Mohd. Solvee, son, Muhammad Sofwan Wazeen, daughter Sofea Adriana and parents, Hassan and Sapura

For their love, understanding, encouragement, patience and moral support.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

IDENTIFICATION OF PROTEIN KINASE INHIBITORY ACTIVITIES FROM *STREPTOMYCES* STRAIN H7372 FOR POTENTIAL USE AS ANTI-CANCER AGENT

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Chairman: **Professor Seow Heng Fong, PhD**

Faculty: **Medicine and Health Sciences**

Aberrations in the phosphatidylinositol 3-kinase (PI3K)/Akt pathway have been found in a wide spectrum of human cancers. Activation of Akt and inactivation of the downstream substrates such as GSK-3β, BAD and Forkhead family (FKHR) proteins are relevant to promote cell survival, proliferation and growth. Another related pathway linked with PI3K/Akt is the Ras/Raf/MEK/ERK, which is known to promote cancer as a result of ras-transformation. The discovery of new drugs targeted at specific molecules of these pathways is a 'hot' field in cancer research. Blocking the constitutively active PI3K/Akt pathway provides a new strategy for cancer therapy. Thus, inhibitors of this signaling pathway would be potential anti-cancer agents. The *Streptomyces* strain H7372 isolated from mangrove soils in Sabah was found to inhibit the Ras/Raf-1 protein interaction in the yeast two-hybrid screening system. The present study was undertaken to determine the cytotoxic effect of H7372 fractionated extract on a breast cancer cell line, MCF-7 and a non-tumorigenic epithelial cell line, MCF-10A and quantitatively measure kinase inhibition, apoptosis induction and cell cycle disruption. The crude

extracts of H7372 were fractionated into eight fractions using reverse phase HPLC. Fraction 5 was found to be the most cytotoxic in an MTT assay. The crude extract and fraction 5 of H7372 were found to exert growth inhibition of MCF-7 at IC_{50} of 15μg/ml and 1.4 μg/ml, respectively. Western blot analyses showed that activated PI3K and Akt (Thr308) but not Akt (Ser473) by stimulation of IGF-I were inhibited by the crude extract and fraction 5 after 72 hours. Interestingly, phosphorylation of Raf-1 (Ser259) and ERK1 were also inhibited by fraction 5, indicated that there is a cross-talk between PI3K/Akt and MAPK pathways. By using the flow cytometry technique, we found that fraction 5 inhibited the proliferation of MCF-7 cell line by causing them to arrest in the G_1 phase of the cell cycle. The induction of growth arrest by fraction 5 was associated with accumulation of cells in G_1 and decreasing cells in S and G_2/M phases. The results were supported by inihibition of cyclin D1 in MCF-7 cells. The apoptosis study showed that fraction 5 but not crude extract was increased the percentage of cells in early apoptotic stage at all concentrations. Furthermore, treatment of MCF-7 cells with fraction 5 resulted in reduction in phosphorylation of GSK-3β (Ser9), phospho-BAD (Ser112) and phospho-FKHR (Ser256). These results could contribute the apoptosis in MCF-7 cell line. Thus, we discovered that fraction 5 of H7372, a naturally occurring microbe, contains an inhibitor of cell proliferation, cell cycle progression and is able to induce apoptosis via the PI3K/Akt and MAPK pathways.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGENALPASTIAN AKTIVITI PERENCAT PROTEIN KINASE DARIPADA *STREPTOMYCES* STRAIN H7372 BAGI KEGGUNAAN YANG BERPOTENSI SEBAGAI AGEN ANTI-KANSER

Oleh

MASRIANA HASSAN

Februari 2007

Pengerusi: **Profesor Seow Heng Fong, PhD**

Fakulti: **Perubatan dan Sains Kesihatan**

Gangguan dalam lintasan phosphatidylinositol 3-kinase (PI3K)/Akt (Protein Kinase B) telah ditemui secara meluas di dalam kanser manusia. Pengaktifan Akt dan penyahaktifan substrat bawahan seperti protein GSK-3β, BAD and Forkhead family (FKHR) adalah berkaitan bagi pemanjangan hayat, penambahan dan pertumbuhan sel. Lintasan lain yang berkaitan dengan PI3K/Akt adalah Ras/Raf/MEK/ERK di mana ia telah dikenalpasti bagi menyebabkan kanser yang disebabkan oleh transformasi raf. Penemuan ubat-ubatan baru bagi sasaran molekul yang spesifik untuk lintasan ini adalah bidang yang hangat dalam kajian kanser. Penghalangan lintasan PI3K/Akt yang aktif berterusan memberi starategi baru untuk terapi kanser. Oleh itu, perencat lintasan isyarat ini akan menjadi agen antikanser yang berpotensi. *Streptomyces* strain H7372 yang diasingkan daripada tanah paya bakau di Sabah telah merencat interaksi protein Ras/Raf-1 di dalam sistem penyaringan 'yeast two-hybrid'. Kajian ini telah dilakukan untuk menentukan kesan sitotoksik oleh ekstrak H7372 yang telah difraksi terhadap sel kanser payudara, MCF-7 dan sel epithelium bukan tumor, MCF-10A, serta mengukur secara

kuantiti perencatan kinase, induksi apoptosis dan pemusnahan kitaran sel. Ekstrak mentah H7372 telah difraksikan kepada lapan fraksi menggunakan fasa berbalik HPLC. Fraksi ke-5 telah dikenalpasti sebagai fraksi yang paling sitotoksik di dalam asei MTT. Ekstrak mentah telah dikenalpasti dapat merencat pertumbuhan pada IC_{50}15μg/ml manakala, fraksi ke-5 pada 1.4 μg/ml. Analisis dari kaedah 'Western blot' telah menunjukkan bahawa pengaktifan PI3K dan Akt (Thr308) tetapi bukan Akt (Ser473) dengan rangsangan IGF-I telah direncat oleh ekstrak mentah dan fraksi ke-5 selepas 72 jam. Fosforilasi Raf-1 (ser259) dan ERK 1/2 juga telah direncat oleh fraksi ke-5 dan ianya menunjukkan bahawa terdapatnya hubungan antara lintasan PI3K/Akt dan lintasan MAPK. Dengan menggunakan teknik 'flow cytometry', kami mendapati bahawa fraksi ke-5 telah merencat penambahan sel MCF-7 dan dengan ini menyebabkan sel tersebut ditahan pada fasa G_1 di dalam kitaran sel. Induksi bagi penahanan pertumbuhan oleh fraksi ke-5 telah menyebabkan pengumpulan sel di dalam fasa G_1 dan mengurangkan jumlah sel di dalam fasa S dan G_2/M. Keputusan-keputusan ini telah disokong oleh perencatan cyclin D1 dalam sel MCF-7. Kajian apoptosis telah menunjukkan bahawa fraksi ke-5 tetapi bukan ekstrak mentah telah meningkatkan peratusan sel pada tahap awal apoptosis bagi setiap kepekatan. Tambahan pula, rawatan sel MCF-7 oleh fraksi ke-5 telah menyebabkan penurunan di dalam fosforilasi GSK-3β (Ser9), BAD (Ser112) dan FKHR (Ser256). Keputusan-keputusan ini boleh menyumbang kepada apoptosis bagi sel MCF-7. Oleh itu, kajian ini telah mengenalpasti bahawa fraksi ke-5 daripada mikroorganisma semulajadi mengandungi perencat bagi penambahan sel, progresi kitaran sel dan juga berupaya untuk merangsang apoptosis melalui lintasan PI3K/Akt dan MAPK.

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Last but not least, I would like to express my heartfelt gratitude to my husband, Mohd. Solvee, thank you for his love and patient, encouragement and endless support throughout this study. Especially thanks to my beloved parents for their understanding and support during the entire study in UPM.

I certify that an Examination Committee has met on 27th February 2007 to conduct the final examination of Masriana Binti Hassan on her Master of Science thesis entitled "Identification of Protein Kinase Inhibitory Activities from *Streptomyces* Strain H7372 for Potential Use as Anti-Cancer Agent" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

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Date: 10 MAY 2007

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

MASRIANA HASSAN

Date: 1st AUGUST 2007

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~	Approximately
%	Percentage
α	Alpha
β	Beta
γ	Gamma
κ	Kappa
μ	Micro
cm	Centimetre
°C	Degree of Celsius
CO_2	Carbon dioxide
g	Gram
kDa	Kilo Dalton
L	Liter
M	Molar
mg	Miligram
min	Minute
mL	Milliliter
mm	Millimetre
ng	Nanogram
rpm	Revolutions per minute
μg	Microgram
μL	Microliter

μm	Micrometer
U	International unit
V	Volt
AMP	Adenosine monophosphate
APS	Ammonium persulphate
ATCC	American Type Cell Collection
BSA	Bovine serum albumin
CDK	Cyclin dependent kinase
DNA	Deoxyribonucleic acid
DTT	Dithioretol
EGF	Epidermal growth factor
ERK	Extracellular signal regulated kinase
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GSK-3β	Glycogen synthase kinase-3Betta
HCl	Hydrochloride acid
HPLC	High performance liquid chromatography
Hsp90	Heat shock protein 90
IDV	Integrated density value
IGF	Insulin-like growth factor
IGF-IR	Insulin-like growth factor-I receptor
ILK	Integrin-linked kinase
IR	Insulin receptor
IRS	Insulin-response sequence